THE FATE OF INDIA INK INJECTED INTO THE BLOOD

II. THE FORMATION OF INTRACELLULAR GRANULES AND THEIR MOVEMENTS

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The vital staining of cells is regarded by many as due to absorption of dye molecules by cell granules, but the appearance of ink granules in the interior of cells has been regarded as the result of phagocytosis. Indeed, ink granules have been introduced into the body for the purpose of testing the functional activity of cells after vital staining. Arnold [1] used dyes and other material in substance for the purpose of staining and ink granules to differentiate between the granules taken up by phagocytosis and the granules colored by vital staining, and concluded that the ink granules often were crowded closely to the cell granules and even covered the latter. Schulemann [2] made a similar observation and J. Koch [3] injected ink into the peritoneal cavity and noted that the ink particles seemed to grow larger within the cells and seemed to be attracted to the cell granules. Evans, Schulemann and Wilborn [4] regard vital staining as the result of a phagocytic process, but Kiyono [5] differs from this opinion while Tschaschin [6] observed that collargol stained a cell granule in the same way as dyes do in vital staining. Kiyono [7] noted, however, that there was precipitation when collargol was introduced into the blood, the granules being taken up by phagocytes. Recently this investigator [8] suggested that both vital staining and phagocytosis may be governed to a certain extent by the same physical laws but to regard them as identical processes, as Schulemann and Evans have done, is not warranted by the morphologic appearances.

No special study has been made of the migration of ink granules in the body except by Kiyono,[9] who explains this migration as the

[1] Virch. Arch., 1899, 157, p. 424.
[2] Arch. f. Mik. Anat., 1911-12, 79, p. 223.
[3] Ztschr. f. Hygiene and Infectionskrankh., 1911, 68, p. 80.
[4] Deutsch. med. Wchnschr., 1914, 40, p. 1508.
[5] Nisshin Igaku, 1914, 4, p. 917.
[6] Folia haemat, 1913, 17, p. 317.
[7] Folia haemat., 1914, 18, p. 149.
[8] Kiyono: Nisshin Igaku, 1918, 8, p. 475.
[9] Kiyono and Murakami: Kyoto Igaku Zassi, 1917, 14, p. 821.

result of excretion of granules by cells into lymph spaces and capillaries. In my work I observed that ink granules are absorbed by granules in leukocytes as well as fixed cells, which is in harmony with what J. Koch found in the case of peritoneal exudate, and furthermore I found that within the cell the ink granules become more irregular and complicated in structure than when they are first taken up.

I did not find any evidence of the exclusion of granules from cells, but I did find evidence of cell degeneration and regeneration in the course of which the granules were subjected to phagocytosis, not only a second but even a third time in such a way that eventually large accumulations would form.

OBSERVATIONS ON THE INK GRANULES WITHIN THE CELLS

In the cells the ink granules vary in size, being larger than the original granule in the ink suspension used for injection. The larger granules in the cells often are from one to one and a half mikrons in diameter. For the sake of convenience ink granules may be classified as follows:

1. The original granule, contained in the ink suspension, is a minute granule of a brownish color, too small to be measured micrometrically, with irregular margins. This granule may persist in the blood for some time.

2. The primary granule is the granule that appears in the cell first. It is usually larger than the original granule but varies much in size, and is spherical with smooth surfaces and densely black as a rule. Some granules seem a little lighter in the center and sometimes they appear unevenly colored. Granules of this kind may appear singly or form conglutinations.

3. The secondary granule is irregular and angular, often forming masses or lumps, developing as a rule some time after the injection, especially when there is a not too rapid degeneration of the cells. These granules may be associated with primary granules, the latter usually being smaller.

4. The tertiary granule is formed from the disintegration of the secondary granule and is smaller and flatter than the other granules, varies much in size and has irregular margins. This granule often looks like dust particles in the cell body or in collections of platelets.

5. By base granule I mean existing cell granules to which the original ink granules are attracted.

In order to observe these different kinds of granules best small doses of ink suspension should be injected. When larger doses are injected many cells become stuffed with granules and aggregations of granules and degeneration of cell occurs so quickly that it is difficult to distinguish various forms of granules.

As indicated, the granules appear first in the cell as primary granules, changing to secondary in a day or in a few days, this change being most noticeable in mononuclears, the polymorphonuclears often being destroyed too early. In accumulations of platelets or cellular débris the granules soon pass into the tertiary form while primary and secondary granules may and often do remain as such. In the endothelial cells of the liver, spleen and marrow the primary granule usually appears spherical and smooth soon after the injection, changing gradually into the secondary form in a few days. The accumulations of granules that eventually develop contain primary, secondary and tertiary forms, the secondary being most numerous. This mixture results from the breaking down of older granules and the new formation of primary granules. In the liver cells the granules are usually of the primary type when seen shortly after the injection, but later they are usually more irregular. In the endothelial cells of the heart and in bone cells the granules are usually of the primary form and seem to remain in this form for some time. In the surface cells and the clasmatocyte of the omentum the granules were usually of the primary type at first, later changing to the secondary type. In the osteoclasts primary and secondary granules appeared, as well as original dustlike particles.

The question naturally arises as to how the granules of India ink injected into the body may grow so large. One might suppose that in the blood plasma the ink would not separate into the original molecules and that some at least would remain as droplets which might give rise to the appearance of a coarser granule within the cell. I have observed this after the injection of large quantities of highly concentrated ink suspension, or when I have killed an animal soon. It is a fact, however, that even the injection of small quantities of highly diluted ink gives rise to the formation of coarser granules.

To study this question more closely I injected slowly 1 c c of a much diluted ink suspension into the portal vein of a rabbit under ether. Immediately after the injection a part of the liver was removed and one hour later another part, bleeding being avoided by compression with a rubber covered forceps. Each piece was fixed immediately.

The part last removed showed larger granules in the endothelial cells, while in the part first removed there were innumerable original granules inside and outside the cells, thus showing clearly that the primary granules grow intracellularly by taking on original granules. Sections from the part first removed also showed that ink granules are attracted to the cell immediately after the injection and adhere to the surface of the cells. In the capillaries, therefore, the ink granules mark the outlines of the cell by arranging themselves around them, the so-called stellar or Kupffer cells being clearly outlined in this way. The attraction of the cells for the granules may be so strong that after injection they disappear quickly to accumulate in the liver and spleen, etc., which become intensely black while the other organs remain practically normal in color.

The reason for the growth of the granules in the cells may be explained as follows: There is in the cells concerned an element that I call the base granule around which the original ink granule becomes regularly arranged by adsorption so that the base granule becomes covered by an ink capsule, so to speak, and this in the primary form of granule. As the primary granule is constructed on the basis of the cell granule, its form will vary according to the shape of the base granule, and it is of course larger than the original ink granule and has a smooth surface. Other points of interest in this connection are that these primary granules frequently have a lighter center than border; in the incompletely developed granule the arrangement of the original granules on the base granule can be made out easily, the surface appearing roughly coated with small masses of varying hue (oil immersion and artificial light), frequently in the form of an arc or horseshoe, whereas the completely developed primary granules show a smooth black surface. When small quantities of ink suspension are injected, incompletely formed primary granules are more abundant.

As stated, the primary granule may change to the secondary form in a few days after the injection and finally into the tertiary. This tendency is more marked in the case of larger granules. The transformation seems to be connected with a change in the base granule which becomes isolated from the rest of the cell by the ink capsule and perhaps may degenerate from other causes as well, and consequently the primary granule collapses in places and finally disintegrates into the tertiary form. The smaller the primary granule the less the change in form as the base degenerates because the wall is thicker

than in the case of larger granules and also because the smaller the diameter the stronger the resistance of the wall as compared with larger granules of the same thickness of wall. This may explain why the smaller primary granules remain longer in the cells or detritus without change of form. In the liver cell proper the granules ordinarily have light centers, incomplete ones occurring, some of which may have well developed outlines. I have also noticed a sort of naked granule in liver cells in sections that were immersed longer in iodin alcohol after fixation in Zenker's fluid. Such granules stain readily with eosin. Arnold,[10] by macerating cells in a solution of iodin and iodid of potassium, was able to differentiate and even to isolate cell granules (plasmosomes). As I have stated, Arnold observed that ink particles would often lie closely to the cell granule, sometimes covering them, thus suggesting that the granules are adsorbed. Examination of leukocytes in a suspension kept at 37 C. for about one hour was found to show clearly the appearances described by J. Koch, the ink granules growing larger within the leukocytes. All these facts indicate clearly that the ink granules accumulate in the cells on the base of preexisting granule-like cell elements, being built up from the small original granules, except rarely as seen in osteoclasts.

THE FATE OF THE INK LEUKOCYTE AND ITS GRANULES

As stated in my previous article, the polymorphonuclears containing ink granules become greatly diminished in number 48 hours after injection, and they disappear entirely between the fourth and seventh day, while the mononuclears though decreasing markedly, are still present. Examination of the blood reveals clearly an extensive destruction of leukocytes and the appearance of large numbers of platelets entangling much débris of tertiary ink granules; for a few days after the injection carefully made films show degenerating leukocytes as well as a remnant containing secondary or tertiary granules and also such signs of degeneration as vacuoles and loss of affinity of the nucleus for stain. In the ink cells vacuoles often seem to develop at the periphery of the ink granule, this being more obvious in the case of the secondary granules or their masses so that it may look as if the granule or mass lies on the surface or in the interior of the vacuole. The vacuole may extend and combine with others to form a large

[10] Arch. f. mic. Anat., 1898, 52, p. 523.

defect in the cytoplasm and finally destroy the cell. In this way granules may drop into the blood stream from loss of support. This vacuole formation may be caused by shrinking of primary granules to secondary, but whether this is the only factor or not is questionable because they grow so extensively that the cell may break up.

The ink cells, with more of the granules, seem to degenerate more quickly than those with less. Thus, the polymorphonuclears which contain more granules decrease rapidly and in 24 hours hardly any can be found with over 10 granules, whereas in the earlier stages polymorphonuclears with more granules are frequent. Before their final disappearance from the blood stream they contain only a few granules, generally one. I never observed any tendency of the granule to fade or disappear gradually, which naturally would be expected on the assumption that they are excreted by the cells. The mononuclear ink cells, however, seem to deviate somewhat from this rule as they continue to appear in the circulation even some time after the injection, being liberated from the liver, spleen, marrow, etc. These places attract ink granules and new ink cells arise from the taking up of liberated granules from disintegrated cells, and these granules it seems are not readily taken up by polymorphonuclears. Hence we do not observe in mononuclears as orderly a decrease as in the polymorphonuclears, but that the cells which contain abundant granules degenerate more quickly than others is also indisputable in the case of these cells. Three or four days after the injection, however, especially after larger dosage, a fairly large number of cells completely filled with granules may be seen in films, indicating that at this stage numerous cells are set free.

Considering the diminution and disappearance of granules from the leukocytes, the question arises whether ink granules are excreted by cells in the same way, as many authors have observed that the dye is excreted in vital staining. As already stated in my previous paper, granules do not appear to be excreted from the body by any organ. In unicellular organisms a substance taken up within the cell body and found indigestible is expelled. It must be questioned, however, whether this mode of excretion occurs in the case of ink granules. The granule that develops in vital staining has long been a subject for discussion. Some authors regard it as a secretory granule; others consider it the result of adsorption of dye by cell elements; a few have found that stained cells may remain in the animal body

even as long as 10 months. In the case of India ink, however, it is difficult to assume that the granules are excreted in the form injected and without cell degeneration. As stated, granules may drop into the blood stream as vacuoles form, but this is the result of cell degeneration and not excretion. Not only does it seem impossible that the granules should separate again in the original form from the dense, compact, lacquer-like shell that forms but, as I have pointed out, the primary granule is converted into the secondary and tertiary kinds, the cell degenerating at the same time. Such granules have never shown any tendency to fade before their disappearance. Hence I conclude that the action of leukocytes is passive rather than active in ridding the body of granules and that the granules are not set free without degeneration or destruction of the leukocytes.

RELATION BETWEEN DESTRUCTION AND REPRODUCTION OF LEUKOCYTES

During 3 to 6 hours after injection of ink suspension, especially in large doses, destruction of polymorphonuclears and mononuclears takes place and a few hours later abnormal cells make their appearance. The abnormal polymorphonuclears that first appear in this stage have azurophil or methylenophil granules. The preexisting leukocytes, excepting perhaps lymphocytes, seem to be destroyed within 6 to 12 hours at the most and to be replaced by a new supply while still in the process of destruction. A more remarkable fact is the morphologic alteration of the polymorphonuclears into a special type, including myelocyte and metamyelocyte. The azuro- or methylenophile granules in these cells increase gradually and at 24 to 72 hours there is present a large number of such special cells with basophile cytoplasm, the size often reaching 15 to 17 mikrons and the granules 1.2 to 1.5 mikrons. It is not clear whether they are immature B granules or not; the larger always exceed the usual granules in size and are often destained in the center as undeveloped ink granules may be. They stain better when treated with concentrated Giemsa's solution for 30 to 60 minutes. The nuclei of this new type of cell also show a peculiarity in that they usually are very coarse and less lobulated, measuring often from 5 to 6 and even 8 mikrons in width. These cells at 72-96 hours, when the cell degenerating process tends to subside, appear with nicely stained nucleus and cytoplasm and decrease gradually to normal size.

Table 1 gives an example of the percentages of this large special type of cell, including myelocytes and metamyelocytes.

Thus degeneration and reproduction take place promptly. New cells are distinguishable in 6 to 12 hours and in 24 to 48 hours practically all the old polymorphonuclears are replaced by a large special type that is succeeded by a return to the normal during the next 72 hours. These special cells were observed by Pappenheim and Szecsi [11] after the injection of saponin and sapotoxin in rabbits, and Pappenheim distinguishes in his atlas [12] the young mother myelocytes and young mikromyelocytes, describing the latter as derived from the former and changing to the usual polymorphonuclear granulocytes through a metamyelocytic stage. The mother myelocytes appear also in chronic leukemia and in atypical acute myeloleukemia (polymorphonuclear mother myelocytes or polymorphonuclear giant leukocytes). The special large type observed corresponds with this polymorphonuclear mother myelocyte, and represents an acute regeneration of leukocytes. This shows us how many leukocytes are destroyed in a short time as that all the reservations in the marrow are sent into the circulation without time to ripen. It is of interest to note that after streptococcus injection I found that all the polymorphonuclears were replaced by this new type within 3 hours.[13]

We have no means of knowing exactly the destined length of life of leukocytes in vivo but in the case of India ink cells, especially the polymorphonuclears, it seems to vary from a few hours to 24 hours. Of course, polymorphonuclear ink cells exist in the blood even 48 hours or so after the injection, but we must bear in mind that after the degeneration of ink cells new phagocytic cells appear which take up the liberated granules. This is clearly shown by the fact that the new type, which appears mainly after the original granules have disappeared from the blood stream, also contains ink granules. The cells having basophilic granules appear as early as 6 to 12 hours, and in 24 to 48 hours they are largely changed to the special type which appears only after large doses of ink and extensive leukocytic destruction. Therefore we may conclude that after ink injection the polymorphonuclear leukocytes up to the appearance of the special new type may be replaced several times by cells of the usual type successively supplied from the reserve in the marrow.

[11] Folia haemat., 1912, 13, p. 25.
[12] Pappenheim: Atlas d. Menschlichen Blutzelle, 1911-12, Supplm., p. 96.
[13] Nagao: J. Infect. Dis., 1920, 27, p. 327.

TABLE 1

PERCENTAGES OF SPECIAL LEUKOCYTES

	Time in Hours After Injection of Ink								
	6	12	24	36	48	72	96	120	
Rabbit 1, 1,450 gm., received 35 c c of ink suspension	3	12	93	...	100	89	12	0	Large cells
	97	88	7	...	0	11	88	100	Small cells
Rabbit 2, 1,350 gm., received 30 c c of ink suspension	2	8	48	72	100	65	5	0	Large cells
	98	92	52	28	0	35	95	100	Small cells

TABLE 2

INK CELLS IN BLOOD OF RABBITS INJECTED WITH BROTH AND SALT SOLUTION SUSPENSION OF KILLED WASHED NONHEMOLYTIC STREPTOCOCCI, 15 DAYS, 3 MONTHS AND 9 MONTHS AFTER THE INJECTION OF INK

	Time in Hours after Injection of Streptococcus Suspension								
	Before	3	6	12	24	47	72	96	
Rabbit 1, 1,450 gm., received 130 c c of streptococcus suspension 15 days after 20 c c of ink had been injected	2	2	14	8	7	5	2	2	Mononuclear ink cells
	0	3	3.3	3	2	2	2	0.02	Polymorphonuclear ink cells
Rabbit 2, 1,950 gm., received 150 c c of streptococcus suspension 3 months after 30 c c of ink had been injected	1	0	2.8	6.7	1.5	0	0.5	..	Mononuclear ink cells
	0	0	2	1.4	0	0	0	..	Polymorphonuclear ink cells
Rabbit 3, 1.980 gm., received 150 c c of streptococcus suspension 9 months after 35 c c of ink had been injected	1	5	6	4	0	1	1	..	Mononuclear ink cells
	0	0.6	0.95	1.4	0.6	0	0	..	Polymorphonuclear ink cells

TABLE 3

INK CELLS IN THE BLOOD AFTER INTRAVENOUS INJECTION OF STREPTOCOCCUS SUSPENSION FILTRATE

	Time in Hours after Injection of Streptococcus Suspension and Filtrate										
	Before	1	3	6	12	24	30	48	72	96	
Rabbit 1, 1,450 gm., received 5 c c of streptococcus suspension 7 days after 10 c c of ink suspension had been injected	1	..	1.2	3.3	-1	1.5	..	2.3	..	..	Mononuclear ink cells
	0	..	0	0.2	1	0	..	0	..	..	Polymorphonuclear ink cells
Rabbit 2, 1,850 gm., received 10 c c of streptococcus filtrate after 15 c c of ink suspension had been injected	..	0	1	9	2	6	7.5	3.7	1.1	0.3	Mononuclear ink cells
	..	0	1	0.7	3.2	3.3	1.5	0.7	0	0	Polymorphonuclear ink cells

THE FATE OF THE ENDOTHELIAL INK CELLS OF THE LIVER, SPLEEN, ETC.

The Degenerative Process.—The degeneration of leukocytes after ink injection is obvious and the next question is whether the endothelial cells of the liver, spleen, etc., also undergo degeneration in the same way. Appearances indicate that endothelial cells may degenerate and fall into the blood stream. In order to study the process more closely the following experiment was made: Four guinea-pigs weighing from 200 to 250 gm., each received a small dose of much diluted ink (0.3 to 1.7 salt solution) intravenously; one was killed at 24, one at 48 and one at 96 hours after the injection by bleeding and the last was killed after one month. Smears were made of the liver and spleen by pressing the slide gently against glass on the cut surface, after wiping off the blood. These smears were stained with hematoxylin and eosin, as well as the Wright stain. The endothelial cells containing ink granules showed marked signs of degeneration, containing vacuoles of various extent often occupying practically the whole cell. In fact, all fields showed many cells largely replaced by vacuoles. At 24 hours the vacuoles were small and increased gradually in size and number up to 96 hours. In this respect the changes correspond to those in mononuclear ink cells in the blood in which the most marked alterations appear 3 or 4 days after a large dose of ink. After one month, when the ink granules had become accumulated in places, the vacuoles and degenerated cells had decreased much, although still present in considerable degree. This shows clearly that the endothelial ink cells also undergo degeneration in increasing degree until the third or fourth day and that as the granules accumulate the degeneration decreases. The vacuoles described in leukocytes and endothelial cells may be secretory in the sense of Metschnikoff and others, but it may be as well a sign of cell degeneration, and the fact that ink cells break down shows that in this case it concerns a degenerative process.

THE TRANSFER OF FUNCTION

Certain authors have tried to use a combined method in vital staining by injection of two independent dyes, but the results have not been clear cut (Goldmann,[14] Schulemann,[15] Arnold, etc.). In such experiments there appeared usually three kinds of cells, two of which contained the granules of each color singly while the other contained

[14] Vitale Karminspeicherung, Jena, 1914, p. 211; Nisshin Igaku, 1914, 4, p. 1113.
[15] Ztschr. f. experiment Pathol. u. Therap., 1912, 11, p. 307.

the granules of each color. Kiyono,[16] however, succeeded in producing violet granules by injection of lithium carmin and trypan blue, mixed as well as separately. Goldmann [17] observed that intravitally stained endothelial cells of the liver were still phagocytic with respect to India ink particles. Arnold proved leukocytes. Recently Ioka [18] distinguished a kind of cell in the ovarian follicles (which he concluded to be a histiocyte) in his study of vital staining of ovary with carmin and the ink. He observed that this cell stained well with carmin and also had a strong phagocytic action on coal suspension; on the injection of a mixture of these substances both appeared in the cell; if he injected carmin first, then coal, the latter was not taken up so freely by the carmin cells; reversing the order of these injections, the results were reversed and appeared even as purely coal-pigment cells.

These results, especially the last, seem to suggest a functional limitation of ink or carmin cells, at least in some degree. To study this question the following experiment was made on seven guinea-pigs of about the same size, using ink and cinnabar suspension.

The cinnabar suspension was made by rubbing a cinnabar stick on an ink stone and filtering through filter paper without centrifugation. The suspension was sterilized by steam as in the case of India ink. The granules of cinnabar are very heavy and fall to the bottom quickly; hence, before using it is necessary to shake for some time. The granules are usually much coarser than those of India ink. The guinea-pigs were divided into 2 series, one of 4 and one of 3. The experiment was made by injecting 0.5 c.c. of each suspension (0.5 each) in a mixture or separately, adding salt solution to make the total quantity injected 2 c c. The first group was treated as follows:

Pig 1 received both suspensions in mixture.

Pigs 2, 3 and 4: Both suspensions were injected separately, first the ink, then cinnabar, after 4 hours into pig 2, after 24 hours into pig 3 and after 96 hours into pig 4.

Each guinea-pig was killed 4 hours after the last injection and sections made of the liver and spleen.

The other group of 3 pigs was injected in the same manner as were pigs 2, 3 and 4 but in reverse order, i. e., the cinnabar first and then ink. These pigs are designated as 2′, 3′, 4′.

Pigs 1 and 2 gave the same result, the cells containing ink and cinnabar. Pigs 3 and 4 showed actively separate color granules; in pig 3 (24-hour interval), however, 3 kinds of granules appeared distributed in a relatively uniform manner in the cells, but in pig 4 (96-hour interval) there was a very irregular distribution of each color. The cells having many ink granules were

[16] Vitale Karminspeicherung, Jena, 1914, p. 211; Nisshin Igaku, 1914, 4, p. 917 and p. 1113.

[17] Berlin klin. Wchnschr., 1912, 40, p. 1689.

[18] Kyoto Igaku Zassi, 1917, 14.

generally poor cinnabar and vice versa, and there were some pure ink or pure cinnabar cells. Pigs 2′, 3′, 4′ gave the same results. This result agrees with Ioka's and also with some of the results with vital staining, 3 kinds of cells appearing after mixed injection. In pigs 1 and 2 the granules of the cells appear to have some affinity for the cinnabar and the ink particles. I observed, however, that in these mixtures of both suspensions the ink particles tend to be absorbed on the surface of the cinnabar particles, the difference in size being quite large. Therefore the result in pig 1 may be explained in this way, but the results in pigs 2 and 2′ enable one to decide definitely. We see from these results that capsulated cell granules attract or adsorb other foreign substances even 4 hours after the injection, though of course these may be influenced by the quantity of preadsorbed stuff. Later, however, this function of the base granules seems to be destroyed by the capsules of the foreign particles injected previously and the particles in the blood stream are adsorbed on other granules; but phagocytic function of the ink or cinnabar cell still remains fairly well marked. This lack of base granules is also of interest in connection with the change of the primary granules to secondary as described. After 4 days, ink or cinnabar cells, especially those stuffed with granules, are damaged greatly and lose their phagocytic action, while the cells that contain few granules still seem able to retain that function in some degree. We must, however, also consider that by this time there are newly produced cells that take up granules liberated from destroyed endothelial cells and leukocytes, and these new ink cells may take up also granules of the subsequent injection. At any rate, the phagocytic action of the endothelial cells may weaken, and on a second injection after some days the number of granules taken up may vary greatly and there may appear even pure ink or cinnabar cells, some arising from the phagocytic insufficiency of the foreign body cell, others from the activity of newly formed cells.

These experiments show that morphologically, as well as functionally, the ink cells, at least those of the leukocyte and endothelial cell nature, degenerate sooner or later after phagocytosis, and that ink or cinnabar granules are liberated again by this process of destruction, but not by secretory or excretory processes.

THE MOVEMENTS OF THE INK GRANULES WITHIN THE BODY

As described in part 1 the ink granules are not discharged from any organ, but wander from place to place until they gradually are stored up in accumulations. This wandering in the earlier stage may depend on the movements of leukocytes and logically they may be carried any place, even outside of the body, especially from foci of inflammation and from wounds or in the saliva, the sputum or other secretory fluids. But so far as I have found, the leukocytal ink cells seem to have little power to migrate, only the ones bearing a small number of granules being able to pass through the capillary walls, though when the walls are damaged they may pass out. In this sense only are the ink granules discharged from the body. This.

however, is not the real movement of the granules in the body, especially in late stages when the ink cells in the blood are few. The real transportation results from the degenerative process of the ink cells and the production of new phagocytic cells. The degeneration and destruction of phagocytic endothelial cells, especially in the early stage, not only discharges ink granules into the blood stream, but also into the lymphatics. The newly formed macrophages, including endothelial and reticular cells, now take up the granules. The appearance of granules in the later stage in connective tissue cells, perithelial cells, bone cells and in lymph nodules, etc., clearly shows the entrance of the granules into the lymph vessels, and their presence in Glisson's capsules in the liver and even in the liver cells indicate a solution of the question. The negative pressure of the thoracic duct influenced by the vena anonyma may act to draw the granules liberated from disintegrating cells into the lymph vessels through some capillary defect. In usual or lower dosage, however, any considerable quantity of granules does not pass at the same time into the lymph space; some are taken up by macrophages in the neighborhood. These new phagocytic cells may weaken and finally disintegrate, granules being sent into the stream, while new cells may be overproduced, with the result that they gradually gather here and there in groups from large accumulations of granules. This may be associated with a similar process in blood capillaries, and in advanced stages the granules may be removed almost completely from general endothelial cells, usually passing from the center to the margin of the acinus; the small accumulations also disappear gradually. In this continual replacing some granules may escape the organ through the hilus by lymph vessels or vein and circulate in general blood, being taken up by some other organ or tissue or return again to the same organ. Even more than a year after the injection the endothelial cells of liver, spleen, etc., often contain small numbers of granules and complete disappearance may not take place at any time, especially if a larger dose was introduced.

The precipitation of granules in advanced stages on the periosteum bone canals, as well as bone cells, especially the latter, situated in only a lymph space, bone lacunae, and the occurrence of ink granules evidently more in the long bones which are rich in marrow, seems to indicate that the granules do not pass into the marrow from other organs but rather pass from the marrow by way of the lymph routes from this organ. This view agrees with the decrease of granules in the marrow in late stages. In the spleen, especially in the malpighian

body as well as in the pulp, which are supposed not to have any lymphatics, the granules still formed accumulations that seem to grow while masses in other organs seem to decrease. This process consequently should proceed only by way of the blood, but we failed to observe any blood stagnation or congestion naturally expected to account for the formation of such accumulations. The accumulations also often occured in the periphery of the central artery, as well as in the outskirts of the spleen body. That granules accumulate in the cardiac and hepatoduodenal nodes as well as in those of the neck and elsewhere is a mystery at present, but that the granules once deposited in endothelial cells in certain organs may be transferred into the lymph stream and circulate in various parts of the body is unquestionable.

The endothelial cells of the liver, spleen and marrow, which receive the granules at the same time, degenerate and some days afterward liberate the granules. At once a heavy loss of cells results, and this gives rise to compensatory phagocytosis by the endothelial cells of other organs. The greater the quantity injected, the greater the amount of phagocytosis and the quicker the destruction of cells. In such cases not only other organs compensate more freely, but newly formed cells are also excessively active. Such rapid changes should give rise to more defects of vessel walls, and all the processes should proceed more quickly than after small doses. At the time of the first injection the endothelial cells are intact and take up the granules. The stomata, if any exist, may pass such small granules easily, but the phagocytic cells hold them back. After phagocytosis of a large quantity, however, this function is lost and the granules may pass relatively abundantly through the wall, though it is necessary to bear in mind that in such large dosage as over 30 c c per rabbit some mechanical distention of the capillary walls may occur.

As stated, Kiyono also attributed this movement of granules chiefly to the lymph stream, but he believed the cells excreted the indigestible foreign body into the lymphatic spaces. I hardly agree to this point, and I would rather emphasize the degenerative processes in the ink cells as an important factor in the movement.

THE RELATION OF THE INK LEUKOCYTES AND STORED INK GRANULES

Polymorphonuclear ink cells disappear from the blood in one week after the injection. The mononuclear ink cells, however, appear

steadily in small numbers, as stated in part 1. During some days after the injection, especially when a dose is given, ink cells seem to appear in the blood by falling from the wall of capillaries containing many ink granules. Later their granule content is very small as a rule, while the endothelial cells of the liver, spleen, etc., and the splenocytes still contain considerable numbers of granules. Why the phagocytic cells do not appear abundantly in blood in the late stages is a question. The endothelial leukocyte or histiocyte (Kiyono) forms a small percentage of cells in the blood in health. Kiyono pointed out that his histiocyte as normal blood element constitutes at most 0.5% of the total leukocytes, but that succeeding injections of carmin solution may increase this number to 2%. This seems to be the case also on injection of India ink.

The problem now considered is how the endothelial ink cells (including splenocytes) behave when there is an acute demand for leukocytes, whether they fall off into the blood stream abundantly or not, and whether the cells in accumulations of many months after the injection will be destroyed.

I have an instance of a rabbit that died from some unknown cause 4 months after the injection. It showed many granules in various forms in the large vessels of various parts of the body, such as kidneys, brain, lungs, etc. This suggests that ink granules precipitated in an organ are not stable but pass into the blood freely. The rabbits were given fairly large quantities of the ink (table 2) 15 days, 3 and 9 months, respectively, before from 130-150 c c broth and salt solution in equal parts were injected peritoneally containing killed washed nonhemolytic streptococci from 24 hour growths, for the purpose of attracting leukocytes. The fluctuation of ink cells in the blood was studied before and after the injection with results given in table 2, which shows that the ink cells in the blood increased markedly until 6 to 12 hours after the injection and then decreased, returning gradually to the usual state. But the granule content of individual mononuclear cells was generally small except in rabbit 1 in which some cells were quite well filled. The most interesting thing in this experiment is the unexpected reappearance of polymorphonuclear ink cells in the blood; there appeared also free ink granules in collections of blood platelets or in cell débris. This shows that the disappearance of polymorphonuclear ink cells in late stages is not due to failure of phagocytosis but rather to disappearance of the granules from the blood, free granules in general circulating blood, if any exist, being few, most of them being taken up by phagocytes. At the same time, it is also indicated that the mononuclear ink cells which appear in blood in later stages come from fixed cells and are not of real hematogenic origin. As stated, endothelial cells or fixed macrophages with large numbers of granules failed to appear in late stages after injection (rabbits 2 and 3), while rabbit 1 showed a few. It seems, therefore, likely that these cells degenerate in loco or soon after falling off and perhaps hardly reach the blood before they liberate the granules. It is possible that a considerable number of cells may meet this fate without being observed.

The polymorphonuclear ink cells in rabbits 2 and 3 disappeared within 6 to 18 hours, while in rabbit 1, in which relatively abundant ink granules appeared free in the blood, they remained for 3 days. This also supports my view that the life of the ink cell is very short and that succeeding generations of cells, as they appear in the blood, create the impression of a longer viability. The mononuclear ink cells in the blood may originate from fixed ink cells and from preexisting or newly formed mononuclear cells, the new cells taking up free granules in the blood.

It appears that by suitable treatment of the animals some time after the injection, sufficient ink granules may be set free so that polymorphonuclear cells again appear in the blood as carriers of ink granules and reproduce the condition found soon after the injection of the ink. Hence it is possible that the storaege of ink granules is not absolutely permanent even under normal conditions, and that the granules may be started into movement again by disturbances, such for instance as arise from bacterial infection. In this connection the results of certain experiments with the products of a nonhemolytic streptococcus are of interest. The streptococcus was grown in 1% dextrose broth for 24 hours when the culture was filtered (Maasen), and injected intravenously in rabbits previously injected with ink suspension. A mobilization of ink cells resulted, most marked about 24-30 hours after the injection, and subsiding within 72 hours. The subcutaneous injection of killed and washed streptococci did not cause the appearance of so many ink cells by far in the blood as the intravenous injection of filtrate of streptococcus cultures or the intraperitoneal injection of broth mixture, but further work will be necessary before conclusions of any value can be drawn. However, it has been shown that fixed ink cells disintegrate naturally and that their destruction may be hastened by certain disturbances and ink granules made to appear again in the blood.

DO INK CELLS MIGRATE?

A mixture of broth and salt solution was injected intraperitoneally at intervals after the intravenous injection of ink suspension, and the peritoneal fluid examined by smears 6-10 hours after the injection. At the tenth hour a quantity of fluid was withdrawn, the leukocytes collected, fixed in formol, embedded in paraffin, and examined in sections. In the smears few ink cells could be found, but in the sections ink cells were found readily, both polymorphonuclear and mononuclear, but the number of granules contained was relatively

small. This result indicates that ink cells do not migrate readily, only cells with a few granules being able to pass through.

Ink suspension alone was injected into the peritoneal cavity, also mixed with broth and salt solution, and many blood films examined 6, 12, and 24 hours later, but practically no ink cells were found in the blood, indicating that a small quantity of ink granules had reached the blood by way of the lymphatics. In the liver and spleen incompletely developed ink granules were found just as they are when minute quantities of ink suspension are injected intravenously. Goldmann, Schulemann and others demonstrated phagocytosis of vitally stained Kupffer's cells in this way in the mouse. Apparently not enough granules enter the blood to give rise to characteristic ink leukocytes, the granules being taken up by the endothelial cells in the liver, spleen and elsewhere, while most of the injected ink is taken up by the cells in the peritoneal exudate and the cells of the omentum and peritoneum and are eventually collected into masses in the omentum or elsewhere after resorption of the fluid. The cells in such masses may disintegrate and secondary, as well as tertiary ink granules, be set free and gradually pass into the blood.

THE ENTRANCE OF INK GRANULES INTO LIVER CELLS

Vital staining of the liver cells proper is difficult and requires many injections (Ribbert,[19] Goldmann,[20] Schulemann, Kiyono, etc.). As stated, ink granules are taken up by liver cells after repeated injections. In one instance the rabbit received 4 c c of ink suspension per kilo and died 4 mouths later from an unknown cause; after death ink granules were found in the liver cells and also in the blood vessels in general. In 2 other rabbits injected with ink daily for 5 and 6 days, ink granules were present abundantly in the liver cells. On the other hand, healthy rabbits injected once only often did not show any ink granules in the liver cells even if the amount injected was fairly large. Kiyono appears to have had somewhat similar results. Sickly rabbits with parasitic disease of the liver nearly always showed ink granules in the liver cells even in a short time after the injection of the usual quantity. It appears therefore that the liver cells under certain conditions take up ink granules, but it is noteworthy that this does not take place uniformly throughout the liver, but only in irregular areas.

[19] Ztschr. f. allg. Physiol., 1904. 4, p. 201.
[20] Beitrage f. klin. Chir., 1909, 64, p. 192.

According to Ogata,[21] the lymphatics of the liver run between the columns of the liver cells and the endothelial cells of the blood capillaries into Glisson's capsule, the liver cells consequently being separated from ink granules in the blood by the endothelial layer and the lymphatic space, the former no doubt restraining them from passing into the lymphatic space. When ink granules come in contact with the liver cells they are taken up by the cells, but evidently special conditions are necessary in order to secure this contact when moderate quantities of ink are injected in the usual way. Kiyono suggests that endothelial cells may pass granules into the lymph spaces by virtue of a special excretory function, but I believe that this transfer may result rather from degeneration of endothelial ink cells. Rossle[22] describes an example of phagocytosis of red corpuscles by liver cells in a case of infection of a man, and he ascribed this phagocytosis to a defect of the capillary wall rather than to diapedesis. The liver of sickly rabbits not only may show many foci to the naked eye, but also in the microscopic section the distribution of the endothelial cells may be very irregular and often groups of cells may form.

In my experiments on the distribution of streptococci in guinea-pigs after injection there was also irregularity in the distribution of the endothelial cells of the liver. In a special experiment, in which a suspension of killed and washed nonhemolytic streptococci was injected intraperitoneally and one animal killed in half an hour, another in one hour, a third after 3 hours, and the fourth after 6 hours, distinct changes in the size and form of the endothelial cells in the liver were found as early as 3 hours after the injection. In order to secure, if possible, more light on the phagocytic action of the liver cells, a special experiment was made on 3 guinea-pigs, this animal usually being free from parasites. Two of the animals were injected intraperitoneally with 70 c c of a suspension of streptococci in broth and salt solution and 20 hours later 2 c c of ink suspension were injected intravenously; one animal was killed 6 hours later, the other was given one more injection of 2 c c of ink suspension at intervals of 5 hours and killed 12 hours after the last injection. The third animal was given 4 c c of ink suspension at the same time and killed 20 hours later. In the first 2 animals the suprarenals, kidneys, lungs, and other organs were fairly black and especially in guinea-pig 2, and sections showed an extensive phagocytosis by the endothelial cells of the capillaries in general, and marked phagocytosis by the liver cells. In both cases there were many granules in Glisson's capsule. The third animal showed only normal conditions to the naked eye and no granules in the adrenal cells. The suprarenal cells of the pig injected with streptococci and once with 2 c c of ink suspension contained some ink granules in the suprarenal cells, while the pig that in addition to streptococci received 2 c c ink suspension twice showed most ink granules in the suprarenals. In both the liver and the suprarenal the distribution of the

[21] Kyoto Igaku Zassi, 1917, 14, p. 821 (cited by Kiyono and Murakami).
[22] Zeigler's Beit., 1907, 41, p. 181.

phagocytic cells was irregular; in the case of the suprarenal there was no phagocytosis on part of the nerve cells in the medulla. According to Kiyono, the cells of the suprarenal are difficult to stain with carmin, success being attained only after repeated injection. So far as I know, ink granules have not been described in suprarenal cells. The suprarenal cells in this case seem to be somewhat degenerated, hence it is possible that the phagocytosis was the result of a special condition in some way.

The fact remains that in the early stages ink granules as a rule do not pass through the capillary wall into the lymph spaces, hence it is necessary first to explain how the granules get into the lymph spaces. We know that various substances on injection may increase the flow of lymph in the thoracic duct; it is clear that if the ink granules were passed on as the result of some such mechanism their distribution would be fairly regular. As the matter stands, the irregular distribution of the ink granules in the liver suggests as the most reasonable explanation of the phagocytosis of granules by liver cells that it is the result of the entrance of granules into the lymph spaces on account of distintegration of endothelial cells and resulting defects in the capillary walls.

THE PHYSICOCHEMICAL PHENOMENA OF VITAL STAINING AND OF ACCUMULATION OF INK GRANULES IN THE CELL

Evans, Schulemann, etc., observed that the absorption of dyes by cells and the duration of vital staining are related to the physical conditions of the solution of the dye, the size of the molecules and the diffusion velocity. Dye solutions that diffuse slowly stain cells slowly but the staining may endure; on the other hand, diffusible dyes stain quickly but the staining soon disappears. Hydrosols of metals are not diffusible and stain the cells with which they come in contact more or less permanently. The taking up of India ink granules by cells has received little consideration from the physicochemical point of view. The microscopic particles in suspension of India ink are not diffusible, and they accumulate in certain cells with which they come in contact without diffusion through the body generally; but, as I have already stated, the accumulation of the granules in cells appears to occur in the same way as in vital staining, namely, by adsorption by preexisting granules in the cells. Hence, the taking up of India ink granules by cells may be regarded as a kind of vital staining or storing up, as Kiyono states it. This view is strengthened by the fact that ink granules once taken up by a cell are not discharged except as the cell undergoes disintegra-

tion when, as pointed out previously, they may be again subjected to phagocytosis.

THE RELATION OF THE INK GRANULES TO OTHER CELL GRANULES, ESPECIALLY THOSE CONCERNED IN VITAL STAINING

There is still much variation of opinion in regard to the nature of cell granules concerned in vital staining. Goldmann regards them as of a secretory nature; Pappenheim and Nakano [23] as plasmasomes; Loele [24] holds that the dye stains lipoid elements in the cells; Tschasthin regards them as in part stained chondriosomes and in part secretory; and Arnold [25] suggests that the granule concerned is derived from the plasmasome. The existence of different granules has been brought out by means of double stain, especially by Kiyono, who distinguishes between carminophil and trypanophil granules as well as granules with affinity for both of these stains. At present it is not clear what kind of cell elements adsorb ink granules and whether they are identical with the granules concerned in vital staining; it is a question also whether the cells that are stained vitally and ink cells undergo the same fate. At present it is clear that the ink particles are adsorbed by cell elements so as to form round capsular masses with smooth surfaces and that these masses later undergo certain changes. It is of interest in this connection to note that Anichikow,[26] by adding hyper- and hypotonic salt solutions, produced somewhat similar changes in the granules of vital staining. I made some experiments with the natural sepia ink of cuttle fish, an ink which consists of much smaller particles and contains tyrosin, and I found that here, especially later, the ink particles eventually form nicely arranged and regular granules in the cells. These granules apparently do not undergo any subsequent changes except a gradual loss of color, thus differing greatly from the ordinary ink granules and corresponding more to the changes described by some observers in vital staining. Other observers have noted that after repeated injections of a vital staining solution the granules in the cells will appear in various forms and that the cells may develop vacuoles and other retrogressive as well as regenerative changes. When great quantities of ink suspension are injected, many cells

[23] Folia haemat., 1912, 14, p. 260.
[24] Folia haemat., 1913, 14, p. 308.
[25] Centralbl. f. Allg. Pathol. u. Path. Anat., 1913, 24, p. 849.
[26] Nisshin Igaku, 1914, 4, p. 917.

become so filled with ink particles that it is difficult to distinguish any definite granules one from the other, but when smaller quantities are injected the ink granules in the cells become better developed. The injection of ink and cinnabar when made at intervals was followed by the formation of pure ink granules and pure cinnabar granules in the same cell, but when ink and cinnabar were injected at the same time the granules in the cells were mostly mixed, indicating that apparently the cell element has the same affinity for these two substances. Usually the ink granules are irregular in size but in the endothelial cells of the liver they are more regular than in those of the spleen. This is true when small quantities of ink are injected; when larger quantities are injected there is more variation in the size and form of the granules in the cells, and in sickly rabbits the granules as a rule are more irregular, suggesting that there may be considerable variation in the original base element in the cells on which the ink granules form.

SUMMARY

Particles of India ink seem to be adsorbed by elements in the cells, probably granules, and a capsule of ink particles is formed which is round and smooth and which I designate as the primary ink granule. In a few days this primary granule becomes irregular, due perhaps to change of the base of the granule, and what I call the secondary ink granule is formed. As the cell disintegrates, these granules become mixed with the débris and may coalesce and form tertiary granules. The smaller primary granules seem to be more resistant than the larger and may remain unchanged for some time.

Most, if not all, cells that take up ink granules undergo destruction quite rapidly; this is associated with the formation of vacuoles, which usually appear first about the ink granules and then gradually extend. Cells that are stuffed with granules disintegrate more quickly than cells that contain only a few granules, and the life of such cells after phagocytosis would seem to last only for some hours.

At the same time that the leukocytes which take up ink granules disintegrate, new polymorphonuclears are supplied by the marrow, and in from 6 to 12 hours after the injection new cells with immature basophilic granules appear in the blood. In 24 to 48 hours later these cells are largely replaced by polymorphonuclear giant leukocytes, myelocytes, and metamyelocytes, and these cells often contain ink granules that have been set free by the destruction of phagocytes.

The phagocytic endothelial cells of the liver, spleen, and other tissues also undergo destruction from vacuolation in the course of some days, but before this time their phagocytic activity, as shown by the results of mixed or separate injections of ink and cinnabar suspensions, becomes greatly reduced. This reduction in activity appears to begin about 24 hours or so after the first injection of ink because after that time new granules often do not appear to be formed in the phagocytic cells.

The ink granules within the phagocytes are set free by the destruction of the phagocytes and not excreted by special cell function. Naturally, phagocytic leukocytes transport granules to some extent and may carry them to the outside of the body, but the main movement of the ink granule is by way of the blood and lymph stream acting on granules freed from disintegrated cells, and such granules may be taken up by phagocytic cells with which they come in contact. Repetition of this process of cell disintegration, liberation of granules, and phagocytosis by new cells eventually results in irregular accumulations of ink masses.

When leukocytes are induced to enter the peritoneal cavity at different stages after the injection of ink, there may again appear in the blood polymorphonuclear and mononuclear ink cells. The latter are probably not merely cells that have fallen off from the endothelial lining or that have been set free from cell accumulation, but come also from mononuclear cells in the blood that are engaged in secondary phagocytosis. The intravenous injection of products of streptococci produce the same result in some degree, but subcutaneous injection of such products has only a slight effect. These results indicate that ink granules may be set in motion again by various procedures, particularly perhaps by bacterial infection.

The injection of chemotactic solution into the peritoneal cavity results in the attraction of cells with few ink granules only; apparently phagocytes stuffed with granules are not able to migrate, and the ink leukocytes that form in the peritoneal cavity do not seem to be able to return to the blood. The ink granules are transferred in small degree only to the blood by way of the lymph in the earlier stages and form primary granules in the liver, spleen, etc.

After such treatment the phagocytosis of ink granules by the endothelial cells of the liver and the suprarenal was followed by the appearance of ink granules in the cell proper of those organs and also in the case of the liver in Glisson's capsule, but the distribution of

these granules was very irregular, suggesting that through some defect of the capillary wall the original ink granule passed easily into the lymph spaces.

The mechanism of the accumulation of ink granules in the interior of cells would seem to be similar to that of vital staining by soluble dye, being governed largely by physical conditions. The granules being nondiffusible, are not discharged from the cells by any excretory process, but liberated only on disintegration of the cell. The base granules which adsorb ink granules appear to be produced in large numbers by the cells after the injection so that the cell becomes filled with ink granules, but in such cases the newly produced granules seem to be more irregular than those present before the injection.

Plate I

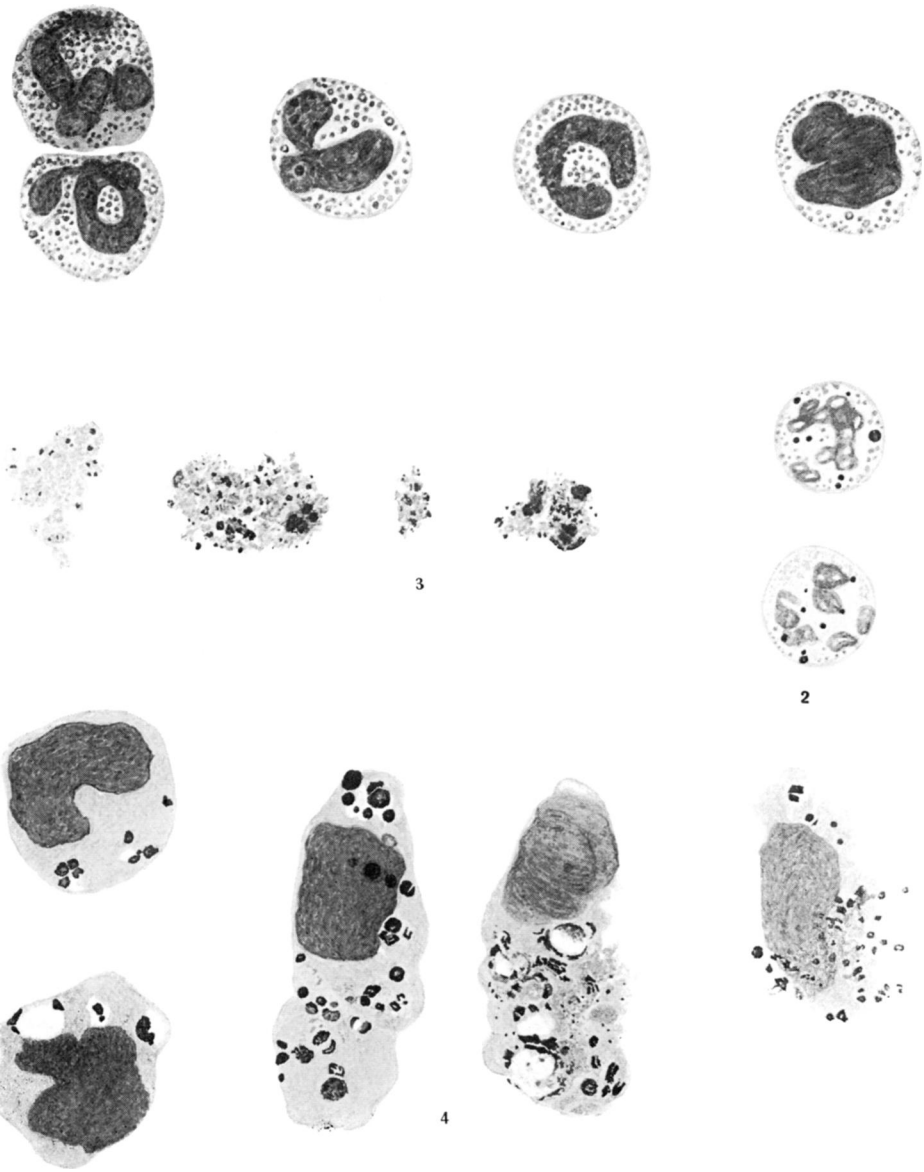


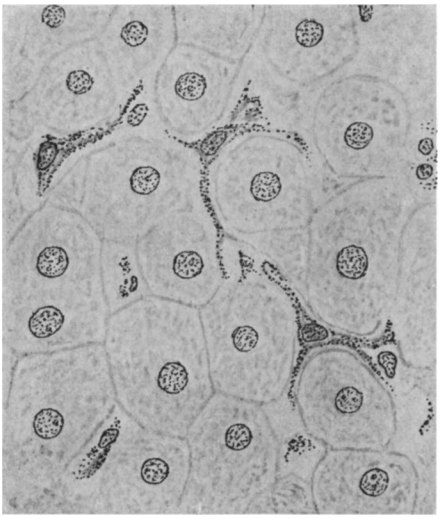
Fig. 1.—Various kinds of polymorphonuclear giant leukocytes with basophilic granules in cytoplasm. Ocular Leitz 4; Spencer oil imm. 1.8.

Fig. 2.—Usual type of polymorphonuclear leukocytes containing mostly primary granules.

Fig. 3.—Various kinds of ink granules, especial tertiary, in débris of cells and accumulation of platelets.

Fig. 4.—Mononuclear leukocytes with various forms of granules showing vacuoles and cell destruction.

PLATE II



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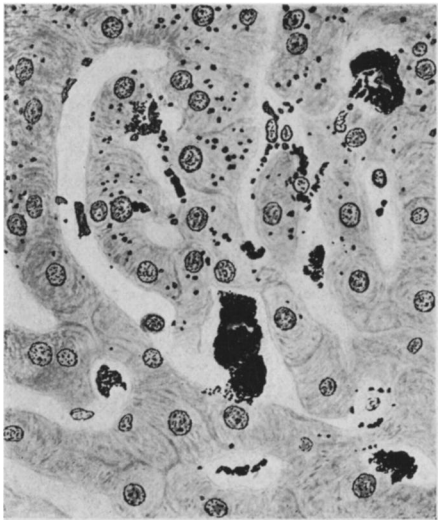

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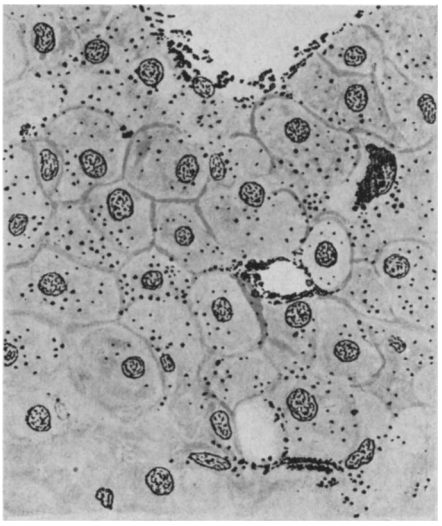

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Fig. 5.—Endothelial cells in the liver of a rabbit immediately after the injection of a small quantity of dilute ink into the portal vein. Leitz ocular 6; Spencer objective 1.8.

Fig. 6.—Phagocytosis by liver cells in a rabbit with coccidiosis. Leitz ocular 2; Spencer immersion objective 1.8.

Fig. 7.—Phagocytosis by suprarenal cells of guinea-pig which had received 2 c c of ink suspension twice following intra-abdominal injection of killed streptococci. Same magnification as Fig. 6.